



Ascorbic acid content and senescence in blueberry (*Vaccinium corymbosum* L.) during storage

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All relevant data are within the paper and its Supporting Information files.

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The authors declare no competing interests.

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Abstract: Blueberry consumption increases because its health properties linked to antioxidants, easy cultivation and profitability. The ability to preserve fruits in controlled atmosphere (CA) allows extending the marketing calendar. The work evaluates parameters linked to the cellular redox state of blueberry fruits, cv. Brigitta, stored at 0°C at different atmosphere regimes (CA1= 10% CO₂, 4% O₂ and CA2= 9% CO₂, 2% O₂, compared to air as control). During storage, quality was assessed by the content of ascorbic acid (AA), antioxidant and index of fruit metabolic status, and of malondialdehyde (MDA), cell membranes oxidative stress and senescence marker; soluble solids content, titratable acidity and dry matter were also determined. Storage in CA increases the blueberries shelf life, particularly at the intermediate times; after 2 months there is a drastic lowering in AA levels and differences among treatments are no longer detectable. Ascorbate is confirmed to be an excellent index of oxidative stress in fruit senescence processes. In control, along with the AA decrease, there is a higher MDA content, in particular up to the intermediate dates. CA2 proves to be the most suitable atmosphere for delaying the senescence process. Titratable acidity and soluble solids remain constant in all samples throughout storage.

1. Introduction

The blueberries (*Vaccinium corymbosum* L.) industry is rapidly increasing because of its healthy properties linked to antioxidant content and its meeting the consumer expectation of healthy food (Gosch, 2003; Kähkönen *et al.*, 2003). Therefore the blueberry growing area is rapidly extending in many regions, taking advantage by the adaptability and ease of cultivation of this crop and its profitability. Blueberry cultivation is well adapted to mountain and hill soil and climate conditions and to oriented organic or environmental friendly agricultural methods with integrated pest management, endowing the growing areas with a benefit of local production and environmental respect. Moreover blueberry, as other small fruit, can be established as small-scale farms giving extra income to family businesses.

The quality of this fruit and the content of health-promoting compounds are influenced by many factors, such as environmental conditions, genetic diversity and degree of maturity at harvest (Ehlenfeld and Prior, 2001; Connor *et al.*, 2002). Blueberry has often been reported to be a highly perishable fruit, thus its commercial value could be strongly affected by storage conditions. Its profitability may take interesting advantage by a storage and shelf life extension that can be achieved by cold storage (0-1°C) and controlled atmosphere (CA) with low oxygen (1-4 kPa O₂) and high carbon dioxide (9-12 kPa CO₂) concentrations (Krupa and Tomala, 2007). After harvest, blueberry quality and product losses are mainly due to dehydration, weight loss, shrivel and fungal spoilage. To understand better the effects of long-term storage on the antioxidant components of blueberry the present study evaluates some parameters related to the cellular redox state of berries of the late cultivar Brigitta, stored at 0°C in different CA regimes.

2. Materials and Methods

Full ripe 'Brigitta' berries were harvested in the Valtellina area (northern Italy, lat: 46.1653333, long: 9.6461111) from 10-year-old plants. The same day of harvest, fruits were put in polyethylene punnets containing about 200 g of fruits each, labelled, weighed and randomly assigned to each of the different storage conditions. Berries were stored at 0°C, 95% relative humidity in the following controlled atmosphere modes: CA1: 4 kPa O₂ and 10 kPa CO₂; CA2: 2 kPa O₂ and 9 kPa CO₂. The control was kept in the air (20.1 kPa O₂ and 0.03 kPa CO₂).

Samples were taken for quality assessment at the following storage times: 0, 12, 33, 47, 61, 76, 94, 110, 132, 147 days.

At each sampling time, 3 punnets per storage condition were removed from the storage and held at -80°C until chemical analysis and quality measurements. Each parameter was determined on 3 replicates, obtaining one fruit sample from each of the 3 punnets per storage condition and time. Dry matter was determined on 50 g of homogenate placed in crucibles and left in an oven at 70°C for 24 hours.

Ascorbic acid was extracted in a 6% (w/v) metaphosphoric acid solution. The amount of 7.5 g of blueberries was homogenized in a mortar with 10 mL of cold extraction medium and centrifuged at 10,000 g at 4°C. The supernatant was transferred into a 25-mL volumetric flask at 4°C. The pellet obtained by

centrifugation was washed with 7 mL of cold metaphosphoric acid solution and centrifuged. The supernatants were combined and brought to a final volume of 25 mL with cold 6% metaphosphoric acid. After filtration through 0.2-µm Nylon filter, a 10-µL sample aliquot was injected onto an Inertsil ODS-3 (5 µm; 4.6 mm × 250 mm) GL Science column at 20°C attached to a Series 200 LC pump (PerkinElmer, Norwalk, CT, USA). The column was eluted with 0.02 M orthophosphoric acid at a flow rate of 0.7 mL/min and ascorbic acid was monitored at 254 nm with a UV-975 intelligent UV-vis detector (Jasco model 7800, Tokyo, Japan). Ascorbic acid was identified by the retention time and quantification was achieved according to the concentration of a corresponding external standard (Sinelli *et al.*, 2008).

The determination of the thiobarbituric acid-reactive-substances (TBARS) content was carried out using a 5% trichloroacetic acid extract. Five grams of mesocarp were homogenized in 25 mL of 5% (w/v) trichloroacetic acid then centrifuged at 4°C at 10,000 g for 30 min. The extract was added to an aqueous solution of 15% (w/v) TCA and 0.5% (w/v) 2-thiobarbituric acid. Samples were mixed and heated at 95°C for 15 min in a water bath, cooled and centrifuged at 4,000 g for 15 min. Samples were then analyzed in a spectrophotometer (Jasco, model 7800, Tokyo, Japan) at 532, 600, and 440 nm. The value of absorbance at 532 nm was purged from the absorbance at 440 nm and at 600 nm due to sucrose and tonon-specific turbidity (Cocetta *et al.*, 2016). TBARS concentration was expressed in MDA equivalent (nmol/g fw) following the equation (Du and Bramlage, 1992):

$$\{[(A_{532} - A_{600}) - (A_{440} - A_{600})(8.4/147)/157\,000]\}10^6$$

For the determination of the titratable acidity (TA), a 5 g sample of homogenized blueberry puree was diluted with 30 mL of distilled water. The TA was measured after 15 sec stirring by titration with 0.1 N NaOH to an end-point of pH 8.3 by a Compact Titrator D (Crison Strumenti SpA, Carpi, Italy). The acidity was expressed as meq/100 g fw.

Total soluble solids (TSS), expressed as percent of soluble solids, were determined by a hand refractometer (Atago mod., N1, Tokyo, Japan) on juice obtained from squeezing the berries.

Analysis of variance was performed by SPSS software, IBM SPSS Statistics 22 (SPSS Inc., Chicago, IL), using general linear model univariate analysis. Sources of variation were time of storage and atmosphere regimes. Significant differences between

means were calculated by Tukey's mean test. Differences at $P \leq 0.05$ were considered as significant.

3. Results

Blueberry fruit cv. Brigitta showed at harvest a percentage of dry matter of 12.52% (Fig. 1). Within the first 47 day storage period, berries maintained in air and in controlled atmospheres exhibited similar dry matter contents (13.45% in control; 12.55% and 12.14% in CA1 and CA2 stored berries, respectively). Thereafter, the level of dry matter increased in control samples until end of storage and was higher than in treated samples. In berries stored in both CA regimes dry matter did not change throughout the entire storage period. At the end of the trial DM accounted for 14.52% of the weight of control fruit and for 11.69% and 11.54% of the weight of CA1 and CA2 stored fruit, respectively.

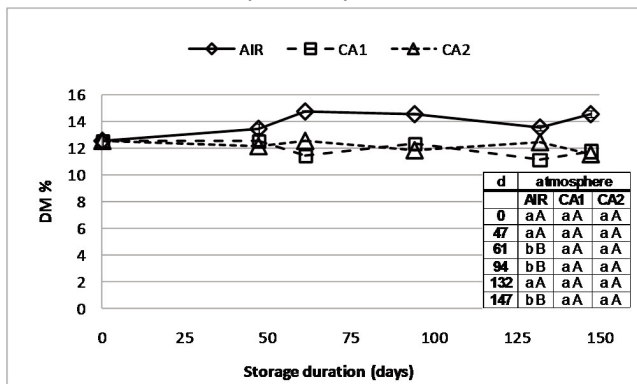


Fig. 1 - Changes in blueberry dry matter during cold storage (0°C) in different atmosphere regimes (Air; CA1= 4 kPa O₂ and 10 kPa CO₂; CA2= 2 kPa O₂ and 9 kPa CO₂). Values are mean \pm SE of triplicate samples. In the table, different lowercase letters indicate significant differences among values after different storage periods at each atmosphere regime and different capital letters indicate significant differences among values at different atmosphere regimes, at each sampling time ($P \leq 0.05$).

The trend of total soluble solids fairly followed the parallel sample variation in dry matter content during storage (Fig. 2). Soluble solid content increased in control fruit during the first storage period, from 11.10% at harvest to 12.70% at 47 days storage, and during the trial it was always higher in fruit stored in air than that in berries stored in CA1 and CA2, except at 132 days of storage. During the trial, berries stored in CA showed no differences in this parameter until day 147, when a decrease was recorded. Comparing total soluble solids at the end of storage period with respect to the beginning, the amounts were 5% higher in berries maintained in air and 12% and 17%

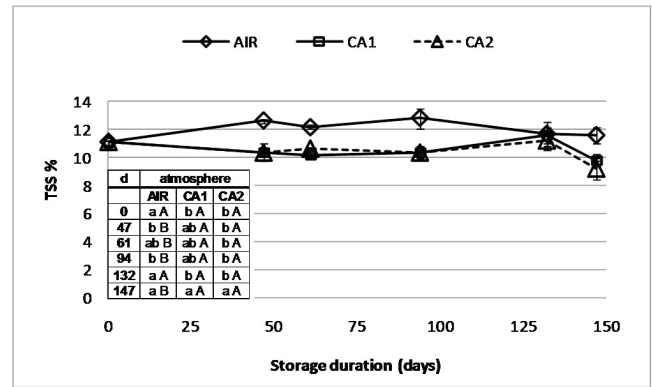


Fig. 2 - Changes in blueberry soluble solid content during cold storage (0°C) in different atmosphere regimes (Air; CA1= 4 kPa O₂ and 10 kPa CO₂; CA2= 2 kPa O₂ and 9 kPa CO₂). Values are mean \pm SE of triplicate samples. In the table, different lowercase letters indicate significant differences among values after different storage periods at each atmosphere regime and different capital letters indicate significant differences among values at different atmosphere regimes, at each sampling time ($P \leq 0.05$).

lower in berries under CA1 and CA2, respectively.

The titratable acidity (11.91 meq/100 g fw at harvest) remained stable in the first 94 days of storage in all the samples and no differences were detectable among treatments (Fig. 3). After this sampling time, TA increased in all samples, reaching a maximum value at day 132 and either maintaining higher values at the last sampling point, in fruit under controlled atmosphere regimes (20.27 meq/100 g fw in CA1 and 19.69 meq/100 g fw in CA2 after 147 days storage), or decreasing, in control, at the end of storage to values not statistically different from those at the beginning of the trial (10.92 meq/100 g fw).

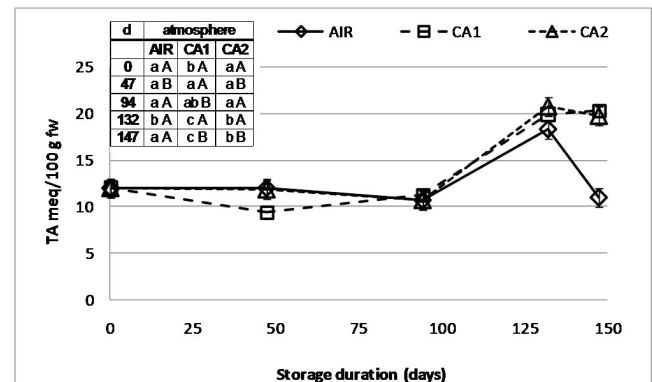


Fig. 3 - Changes in blueberry titratable acidity during cold storage (0°C) in different atmosphere regimes (Air; CA1= 4 kPa O₂ and 10 kPa CO₂; CA2= 2 kPa O₂ and 9 kPa CO₂). Values are mean \pm SE of triplicate samples. In the table, different lowercase letters indicate significant differences among values after different storage periods at each atmosphere regime and different capital letters indicate significant differences among values at different atmosphere regimes, at each sampling time ($P \leq 0.05$).

Ascorbic acid content (0.35 mg/100 g fw at harvest) increases after the first 12 days of storage in control fruit by 377% and in CA1 and CA2 stored samples by 218% and 239%, respectively (Fig. 4). After this sampling time the levels in the control progressively declined reaching the minimum level of 0.18 mg/100 g fw, while ascorbate levels remained higher in the berries stored in both CA regimes until the day 61 (1.31 mg/100 g fw in CA1 and 1.39 mg/100 g fw in CA2). From that date the ascorbic acid content of treated fruit decreased drastically and after the sampling time at 94 days storage ascorbate content was no longer detectable in any sample.

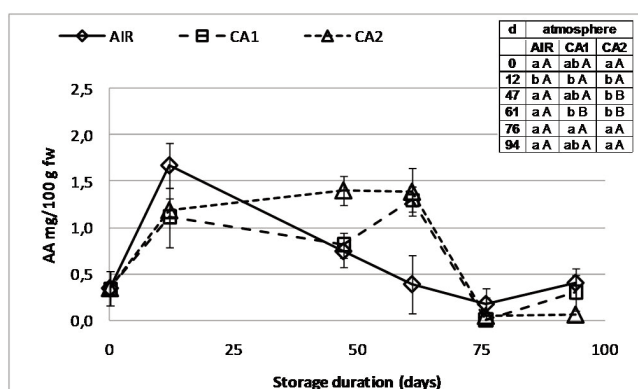


Fig. 4 - Changes in blueberry ascorbic acid content during cold storage (0°C) in different atmosphere regimes (AIR; CA1= 4 kPa O₂ and 10 kPa CO₂; CA2= 2 kPa O₂ and 9 kPa CO₂). Values are mean \pm SE of triplicate samples. In the table, different lowercase letters indicate significant differences among values after different storage periods at each atmosphere regime and different capital letters indicate significant differences among values at different atmosphere regimes, at each sampling time ($P \leq 0.05$).

In control fruit TBARS levels (197.95 nmol/g fw at harvest) decreased at the first sampling date and then increased markedly (+33%), reaching a maximum after 33 days of storage (Fig. 5). TBARS decreased over time, starting at 94 days of storage, from 222.10 nmol/g fw to 138.18 nmol/g fw at the end of storage. In CA stored berries, the trend of TBARS content progressively decreased, but no significant differences were observed compared to harvest date, except for fruit stored in CA1 for 61 days. At the end of the trial, TBARS levels reached values of 170.32 and 143.9 nmol/g fw in CA1 and CA2 stored berries, respectively.

4. Discussion and Conclusions

Dry matter content, linked to water and weight loss and to metabolic activity, is an important factor

affecting blueberries storage life. As blueberries have a high surface-to-volume ratio, they are prone to water loss. The similar values showed by all the samples during the first 47 day of cold storage are in accordance with the research findings of Duarte *et al.* (2009). They reported a markedly low (0.9%) weight loss found in 'Brigitta' fruit at 48 d of cold storage, regardless of the gas mixture used, either air or CA.

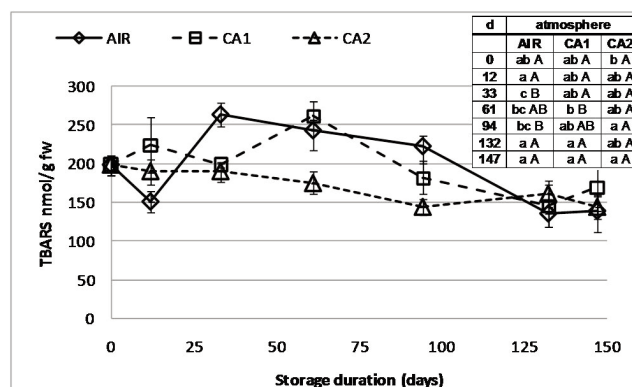


Fig. 5 - Changes in blueberry malondialdehyde level, expressed as TBARS content, during cold storage (0°C) in different atmosphere regimes (AIR; CA1= 4 kPa O₂ and 10 kPa CO₂; CA2= 2 kPa O₂ and 9 kPa CO₂). Values are mean \pm SE of triplicate samples. In the table, different lowercase letters indicate significant differences among values after different storage periods at each atmosphere regime and different capital letters indicate significant differences among values at different atmosphere regimes, at each sampling time ($P \leq 0.05$).

The higher percentages of dry matter determined in control starting from the sampling date after 61 days is due to the greater loss of water sustained by the berries kept in the air with respect to the samples stored in CA1 and CA2. In contrast to our data, Alsmairat *et al.* (2011) reported an effect of CA on moisture loss, yielding a 13-fold difference between the 0% CO₂/21% O₂ treatment (0.25% weight loss) and the 19% CO₂/2% O₂ treatment (3.3% weight loss). The authors reported that the greater weight loss for the highest CO₂/low O₂ treatment could be associated to physical causes, e.g. the greater flux of dry gas through the CA chambers, and secondly because high levels of CO₂ can stress blueberry fruit. On the other hand, in our trial a slight impact on moisture loss in control fruit may stem from similar physical causes, because the CA chambers are smaller than the storage room of control fruit and also because control storage condition could be more stressful to berries than the CA.

Blueberry fruit cv. Brigitta at harvest showed a total soluble solids content and titratable acidity according to other research data (Hancock *et al.*,

2008; Gündüz *et al.*, 2015).

Soluble solids content and titratable acidity level are important quality parameters that account for the flavor of fruit.

Total soluble solids of control samples increased by 13% during the first storage period of 47 days, according to Chiabrando *et al.* (2009) who recorded changes in soluble solid content in cv. Coville, with significantly higher values after 28 days of storage in air at 0°C. This increase in control samples cannot be completely explained on the basis of water loss by transpiration and could be more closely associated to the final events of the ripening process. Although blueberry is expected to stop sugar accumulation once the fruit is picked (nonclimacteric behavior) and does not have starch to support soluble sugar synthesis after harvest, an increase in carbohydrate levels may be a consequence of cell wall degradation. In fact, during this storage period of 47 days no significant increase in dry matter linked to a change in moisture content is observed in control fruit. Thereafter, soluble solids content did not change until end of the trial. Similar research findings were reported on cultivars Brigitta (Duarte *et al.*, 2009), Bluecrop and Ivanhoe (Beaudry *et al.*, 1998), Burlington (Forney *et al.*, 2003). In CA stored fruit, soluble solids content shows similar values throughout the storage period until the last sampling time, when it decreased significantly both in CA1 and CA2 stored berries. The steady values of total soluble solids shown by the samples stored in CA starting from the beginning of cold storage can be due to a stronger effect of CA on fruit metabolism compared to air-stored fruit.

Titratable acidity showed no changes in all samples stored up to 94 days. This is in accordance with previous studies carried out by Chiabrando *et al.* (2009) on 'Bluecrop' and 'Coville' (fruit stored for 35 days in air) and Smittle and Miller (1988) on rabbiteye blueberry (fruit stored for 42 days at different atmosphere regimes) which showed titratable acidity not to be affected by storage duration or atmosphere. On the other hand, after this storage period of 94 days, the parameter increased markedly at day 132 and then remained stable in CA stored fruit until end of the trial. Schotsmans *et al.* (2007) reported a significant increase in titratable acidity during CA storage (2.5% O₂, 15% CO₂) of rabbiteye blueberry 'Centurion' fruit from 35 days onwards.

In contrast to CA stored fruit, in control fruit titratable acidity decreased after the peak at day 132 to levels similar to that recorded at harvest.

According to this trend, Harb *et al.* (2014) found no significant differences in titratable acidity between air-stored and CA stored blueberries cv. Duke after 4 weeks storage, but a 8 week storage period resulted in significant higher level of titratable acidity in CA stored fruit respect to control fruit.

Increased titratable acids contents of blueberries did not correspond proportionately and cannot be explained with declining moisture content of the berry, rather may be linked to the onset of senescence and to the release of additional acids associated with softening and cell wall breakdown (Proctor and Peng, 1989). The higher levels in titratable acidity at the end of storage in CA stored fruit compared to control could indicate that such fruit still had more reserves remaining whereas the reserves for the air-stored fruit were partially depleted.

Ascorbic acid is an antioxidant which concur to fruit quality and it is a marker of the metabolic status of fruit. Growing season, location, agricultural practices, cultivar and crop ripeness affected to varying degrees ascorbic acid levels at harvest. The levels of 1.7 mg/100 g recorded at full ripeness in control fruit of 'Brigitta' were low compared to some other research data (10.2 mg/100 g reported by Golding *et al.*, 2014; 13.6 mg/100 g reported by Kozos *et al.*, 2014) but similar to other studies (4.8 mg/100 g reported by Spinardi *et al.*, 2009; 2.6 mg/100 g reported by Sinelli *et al.*, 2008).

During the first 12 days of storage ascorbic acid levels increased markedly and reached a maximum in air-stored fruit, when the ripening process is completed. The significant increment in ascorbic acid content may rather be due to a partial disassembly of cell wall polysaccharides (Davey *et al.*, 1999; Gilbert *et al.*, 2009; Cruz-Rus *et al.*, 2011) than to the activation of the ascorbic acid biosynthetic pathway. It could also be related to a more efficient ascorbic acid recycling pathway (e.g. enzymes of the ascorbate-glutathione cycle), which plays an important role in the response and adaptation to the stress (Stevens *et al.*, 2008). After this storage period, ascorbic acid content in control samples progressively diminished. This steady decrease could be due to less effective recycling of ascorbic acid or the presence of factors that promote oxidation of the ascorbic acid pool such as the enzymatic activity of ascorbate peroxidase (APX) or the direct interaction with reactive oxygen species (ROS). Zhou *et al.* (2014) found a progressive increment in APX activity in blueberries until 45 days of cold storage in air and a higher level after 60 days compared to the beginning of storage. Throughout

storage, they found also a steady increase in superoxide radical production rate and hydrogen peroxide content.

In contrast to the decrease recorded in control fruit after the first sampling time, ascorbic acid content did not change in CA stored fruit up to 61 days, when the levels were significantly higher than in control fruit. This evidence is partially in contrast to a previous report on cv. Bluecrop (Harb *et al.*, 2010) that indicate that a marked loss in ascorbic acid over the entire storage period of 6 weeks occurred under all storage conditions (ranging from 0% CO₂, 18% O₂ to 24% CO₂, 2% O₂) and stated that increasing CO₂ and/or decreasing O₂ partial pressures within the storage atmosphere did not decisively change this loss. On the other hand, Harb *et al.* (2010) found that storing fruit under low O₂ combined with high CO₂ level (up to 18%) resulted in better preservation of ascorbic acid and that the highest CO₂ level (24%) was injurious and resulted in lower ascorbic acid content.

The malondialdehyde (MDA) content (determined as TBARS level) of 198 nmol/g fw at harvest is similar to the levels reported on blueberries (Zhou *et al.*, 2014), on pears (Cocetta *et al.*, 2016) and grapes (Xu *et al.*, 2009). The levels of MDA were coherent in part with the ascorbic acid levels, and reflected more the differences between the various treatments than the trend of a single treatment during the storage period. In control fruit the decreasing trend of ascorbic acid levels during storage is accompanied with a significant higher malondialdehyde content than that recorded in fruit maintained in CA₂, up to 94 days of storage. Malondialdehyde is considered a biochemical marker of lipid peroxidation of membranes. This data demonstrates a greater oxidative damage to the cellular components of fruit in a natural atmosphere. The drastic decrease in the ascorbic acid content in the second part of the storage period is not in fact accompanied by an increase in MDA levels. This could be due to the onset of senescence processes associated to a loss of cellular integrity and compartmentalisation, as a result of the drastic lipid peroxidation of cell membranes caused by ROS accumulation, and therefore not to a recovery of oxidative stress. The analysis of the TBARS content shows a positive effect especially of CA₂ on the fruit, in which the senescence process is delayed and there is no increase in the levels of this parameter.

The present work confirms the ascorbic acid content as a parameter closely associated to the cellular metabolic state and therefore an excellent index of

oxidative stress that intervenes in the processes of senescence of the fruits during storage. Moreover, the blueberry 'Brigitta' storage in Controlled Atmosphere, mainly at low O₂ level (CA₂), shows a positive effect in delaying the fruit senescence as demonstrated by the reduced content of malondialdehyde.

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